Biochemical and Biophysical Research Communications 440 (2013) 300–305

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Direct inhibition of cell surface ephrin-B2 by recombinant ephrin-B2/FC

Hu Xiaodong [a,1], Huang Zhen [a,*], Sun Min [a], Cui Zhiming [b], Ji Hongyan [a], Zhang Chong [a], Tan Xuefeng [a], Jin Guohua [a,*]

[a] Department of Anatomy and Neurobiology, The Jiangsu Key Laboratory of Neuroregeneration, Nantong University, Nantong, Jiangsu Province 226001, China
[b] Department of Spinal Surgery, The Second Affiliated Hospital of Nantong University, Nantong, Jiangsu Province 226001, China

ARTICLE INFO

Article history:
Received 26 August 2013
Available online 25 September 2013

Keywords:
Endocytosis
Ephrin-B2
Neural stem cell
Neurogenesis
Signal transduction

ABSTRACT

First messengers and viral transfection are the two most common ways to stimulate cells for signal output, although their applications are limited. We investigated mechanisms of inducing neural stem cell differentiation using recombinant ephrin-B2/Fc and found that it acted as a ligand and inhibited endogenous ephrin-B2, which maintenance of the neural progenitor cell state, by direct interference. Our results showed the movement of ephrin-B2/Fc within the cell and indicated that it recycled to the plasma membrane surface, revealing a possible pattern of ephrin trafficking. Our results also serve as proof of concept for the reconstruction of the intracellular domain of ephrin using an artificial receptor to direct input signals in future studies.

1. Introduction

The classical cell signaling pathway consists of the binding of a ligand to a receptor, resulting in the transmission of a signal to the inside of the cell. This process is regulated by several factors that mainly affect the receptor, including the regulation of gene transcription, proteasome-mediated degradation [1] and signal endocytosis [2]. These factors can affect the efficiency of ligand binding.

Ephrins, which contain ephrinA1-6 and ephrinB1-3, are glycophosphatidylinositol-linked (ephrin-A) and transmembrane (ephrin-B) ligands that bind eph receptors on juxtaposed cell surfaces [3]. Ephrin/eph is bidirectional signaling, both of them are able to signal into the cells that express them [4]. A portion of receptor of ephrin-eph could recycle to the plasma membrane after endocytosis [5]. However, the trajectory of the movement of ephrin-eph as ligand is unclear. Moreover, its functions remain unknown.

Recent studies have indicated that Ephrin-B2 has involved in neural stem cell behavior. For instance, disrupted ephrin-B2 forwarding signaling could induced SVZ neural stem cell proliferation and stop migrating in adult subventricular zone [6]. In addition, ephrin-B2 forwarding signaling could also regulated adult hippocampal neurogenesis by eph-B4 receptor on NSCs [7]. However whether the movement of ephrin-B2 as ligands would impact on neurogenesis remain unknown. We used ephrin-B2/FC to treated NSCs, and found that it could endocytosis into cell surface as ligands and then recycle to the membrance surface to inhibit endogenous ephrin-B2, which could promote the differentiation of neural stem cells. To our knowledge this is the first report linking movement of ligands with cell behaviors.

2. Materials and methods

2.1. Primary cell culture

The method used was as described previously [8]. Briefly, NSCs isolated from pregnant Sprague to Dawley rat embryos at E14 were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (DEMEM/F12, 1:1, v/v) (GIBCO) and 2% B27 (GIBCO) supplemented with 20 μg/ml basic fibroblast growth factor (bFGF) and 20 μg/ml epidermal growth factor (EGF) (Sigma) for 4 days.

2.2. Assay for rapid ERK activity

NSCs were cultured in 6-well plates and after 24 h anti-human IgG/Fc (Sigma) was added to pre-clustered ephrin-B2/Fc (Mouse, Sino Biological) at a fixed ratio of 0.1x and incubated for 1 h. Cells were then treated with 2.5 μg/ml ephrinB2-Fc or Phosphate-Buffered Saline (PBS) for 5, 10, or 30 min, and lysed in lysis buffer (Beyotime). Immunoblotting primary antibodies used were anti-phosphorylated ERK (1:2000, Sigma) or anti-ERK (1:8000, Sigma) with HPR-conjugated secondary antibody (1:2000, Millipore).

* Corresponding authors.
E-mail addresses: huangzhenl@ntu.edu.cn (H. Zhen), henryntu@hotmail.com (J. Guohua).
[1] Present address: Department of Anatomy, Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

0006-291X/$ - see front matter Crown Copyright © 2013 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.070

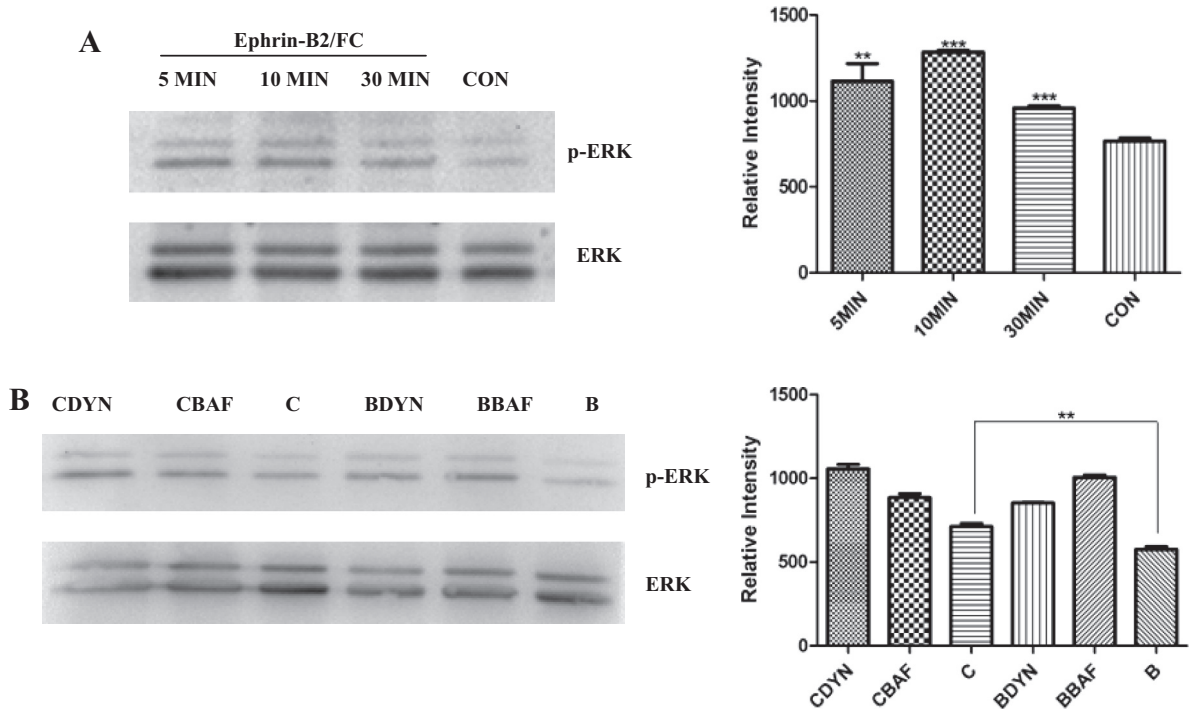

Fig. 1. The expression of ERK in NSC induced by ephrin-B2. (A) A rapid treatement by ephrin-B2/FC. (B) Long-term (24 h) treatment by ephrin-B2/FC. (CDYN: control + dynasore, CBAF: control + bafilomycinA1, C: control, BDYN: ephrin-B2/FC + dynasore, BBAF: ephrin-B2/FC + bafilomycinA1, B: ephrin-B2/FC).

2.3. Assay for 24 h ERK activity and ephrin-B2 protein analysis

Cells were pretreated with 80 μM dynasore (Sigma) or 0.5 μM bafilomycin A1 (InvivoGen) for 10 min and then treated with 2.5 μg/ml ephrin-B2/Fc or DMSO for 24 h. Cells were then assayed with the procedure described above for the rapid assay. Antibodies used were anti ephrin-B2 (1:700, Sigma) and anti β-Actin (1:2000, Millipore).

2.4. RNA interference and efficiency analysis

Three small interfering RNA (siRNA) duplexes specific for rat EFNB2 were designed by GenePharma Co., Ltd. A negtive control was also synthesized (5′-GCGACGAUCUGCCUAAGAUdTdT-3′ and 5′-AUCUUAGGCAGAUCGUCGCdTdT-3′), and carboxyfluorescein mixed isomers [FAM (6-carboxyfluorescein)] were used as indicators of transfection efficiency. Transfection of siRNA was performed using the siRNA-Mate™ Kit (GenePharma) according to the manufacturer's instructions. After 6 h, the cells were fixed and stained with Hoechst 33,342 (1:2500, Sigma) to determine transfection efficiency. Confocal laser microscopy (FV10i, Olympus) revealed that the transfection efficiency exceeded 95%. To assess the inhibition efficiency, the cells were culture in 6-well plates for 24 h after transfection and examined by real-time PCR. One of the siRNA segments, (5′-GACAGAUGCCACUAUUAAGATT-3′ and 5′-UCUUAAUAGUGCAUCUGUCTT-3′) showed silencing of more than 60% of efnB2 mRNA. Therefore, this segment was used in the subsequent experiments.

2.5. Tuj fluorescent intensity assay

NSCs were cultured in 24-well plates (500 ml DMEM +2% B27) at a density of 4×10^4 cells per dish for 24 h. Then, 100 ml DMEM were removed and replaced by 100 ml Opti-MEM (Invitrogen) containing siRNA and the silencing transfection kit, 2.5 μg/ml ephrin-B2/Fc, or 0.5 μg/ml ephrin-B2/Fc; an untreated control was also included. After four days, the cells were stained by chicken anti-Tuj (1:1000, Millipore) primary antibodies and goat anti-chicken Alexa 568 IgG (1:1200, Invitrogen) secondary antibodies. Images were captured by confocal laser microscopy (FV10i, Olympus), and analyzed by FV10-ASW 2.1 viewer software.

2.6. Real-time polymerase chain reaction

RNA was prepared from cells lysed in Trizol (Pufei Biotech). cDNA was prepared from RNA using the Quantscrip RT kit (TIANGEN). Primers used were as previously described (*efnb2* [9], GAPDH [10]). Real time quantitative RT-PCR was performed using the Sybr green premix kit (TIANGEN) and assays were performed in triplicate.

2.7. Cell surface labelling of immunofluorescence staining

This assay was a modification of a previously described procedure [11,12]. Cells were plated at a density of 4×10^4 per dish in 24-well plates in DMEM/F12 and 2% B27 for 24 h. Cells were then treated with 80 μM dynasore (Sigma) or 0.5 μM bafilomycin A1 (InvivoGen) or DMSO, with each group consisting of four wells. After 10 min, ephrin-B2/Fc (2.5 μg/ml) (Mouse, Sino Biological), which was pre-clustered by anti-human IgG/Fc (Sigma) for 1 h at 4 °C, was added to the plate and incubated for 1 h. We then used 0.2 M acetic acid and 0.5 M NaCl acid buffer to remove the cell-surface-bound FC-fusion proteins for 10 min on ice and the procedure was repeated three times. Cells were gently washed three times with PBS for 10 min and then fixed with 3.7% paraformaldehyde for 10 min at 4 °C. After rinsing three times with PBS, Image-iT™ FX signal enhancer (Invitrogen) was applied, which corrects for nonspecific interactions of a wide variety of fluorescent dyes with the cell, for 30 min. After rinsing thoroughly, blocking was performed with 3% bovine serum albumin (BSA) (Amresco) and 10% goat serum (Millipore) for 1 h at 37 °C, and then incubated at room temperature to continue blocking for 1 h. Primary antibodies

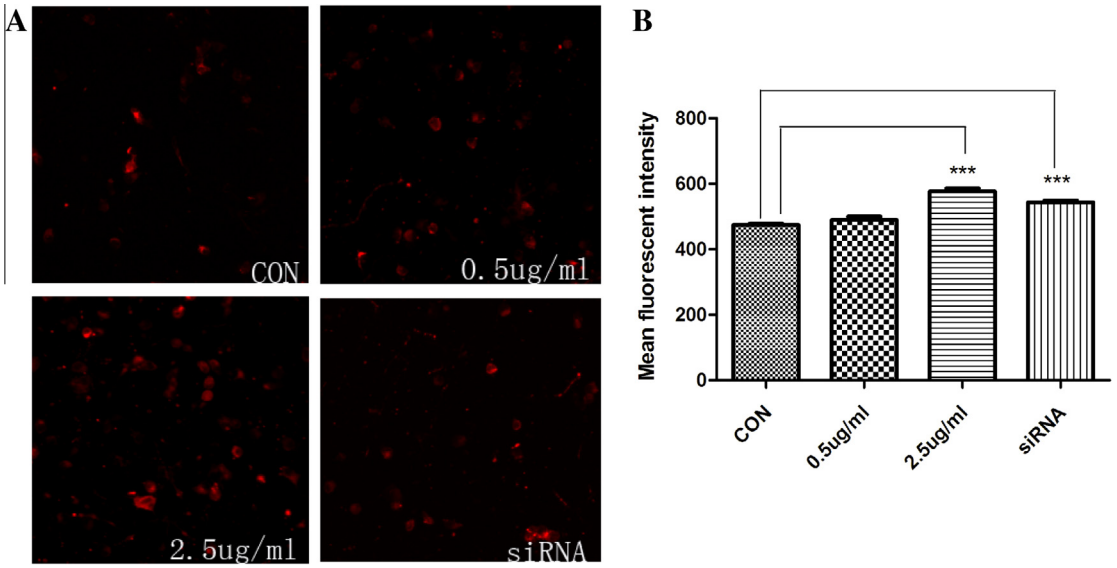

Fig. 2. The intensity of Tuj expression. (A) The NSCs were treated PBS, 0.5 μg/ml ephrin-B2/FC, 2.5 μg/ml ephrin-B2/FC, siRNA transfection kit respectively for 4 days, and stain by Tuj. (B) The fluorescent intensity of Tuj were determined by FV10-ASW 2.1 Viewer software. Both 2.5 μg/ml and siRNA interference were higher intensity than control, while 0.5 μg/ml had no difference with control. Data are the average ± S.E.M of four independent experiments. $^{***}p < 0.001$ (Student's *t* test) (CON: control, 0.5 μg/ml:0.5 μg/ml ephrin-B2/FC, 2.5 μg/ml:2.5 μg/ml ephrin-B2/FC, siRNA:siRNA interference).

against ephrin-B2/Fc (rabbit anti-mouse ephrin-B2 produced from the ephrin-B2/Fc immunogen, 1:200, Sino Biological) were applied for 1 h at room temperature. After washing, secondary antibodies (goat anti-rabbit IgG, 1:1000, Invitrogen) were incubated for 30 min at room temperature. After washing, samples were mounted using the antifade kit (Beyotime). Images were captured by a confocal laser microscope (FV10i, Olympus).

2.8. Accessed cell surface labelling by ELISA

The assay was as described previously. Most of the process was the same as that used for labeling by immunofluorescence staining, with Tris-buffered saline (TBS) instead of PBS, and the secondary antibody was HPR-conjugated (1:700, Millipore). After rinsing thoroughly, antibody binding was visualized by adding 0.25 ml of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Beyotime). After 30 min, 0.1 ml of the substrate was removed to a 96-well plate, and color intensity was measured at 370 nm in a microplate reader (Synergy2, BioTek).

2.9. The EEA1 staining

The cells were plated at a density of 4×10^4 in a 24-well plate. After ephrin-B2/Fc treatment for 1 h, cells were fixed with 4% paraformaldehyde for 10 min at 4 °C. After rinsing thoroughly, cells were treated with 0.3% Triton X-100 solution on ice for 5 min, rinsed three times, and then blocked with 3% BSA and 10% goat serum for 2 h at room temperature. Primary antibodies used were mouse anti-EEA1 (1:500, Sigma) and rabbit anti-human IgG/Fc (1:1000, Sigma) for 1 h at room temperature. Secondary conjugates used were goat anti-mouse Alexa 488 (1:800, Invitrogen), goat anti-rabbit Alexa 594 (1:1200, Invitrogen) for 1 h at room temperature. Images were captured by a confocal laser microscope (FV10i, Olympus).

2.10. Statistics

Statistical analysis was carried out using GraphPad software (GraphPad Prism v4.0). Data are presented as means ± SEM. Significant differences between and within multiple groups were examined using ANOVA, followed by Tukey's post hoc multiple comparison test and Student's *t*-test were used for two group values. Significant differences of means are indicated as $^{*}<0.05$, $^{**}<0.01$, and $^{***}<0.001$.

3. Results and disscussion

Previous studies showed that ephrinA induced neural stem cell (NSC) differentiation were attibutable to Ras-MAPK acivities, whereas ephrin-B2 treatment abolishes basal ERK phosphorylation [8]. However, ephrinB2-Fc treatment for short periods (5, 10, 30 min) resulted in the activation of ERK (Fig. 1A), whereas sustained (24 h) ephrin-B2/Fc treatment abolished ERK phosphorylation. (Fig. 1B). These results were different from ephrinA1 treatment, show that the MAPK pathway is not the main way of ephrin-B2 induce neurogenesis. Due to the characteristic of endocytosis of eph [3], we next asked if endoytosis had an effect on MAPK pathway. Dynasore, an inhibitor of clathrin/dynamin-dependent endocytosis [13], did not decrease ERK phosphorylation when co-treated with ephinB2-Fc. Furthermore, Bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase that inhibits receptor recycling [14] showed similar results than dynasore treatment in that ERK phosphorylation was not abolished compared with the group treated with ephrin-B2/Fc alone (Fig. 1B). These results indicate that the endocytosis of ephrin-eph inhibits ERK phosphorylation.

Recent studies have shown that ephrin-B1 reversed signaling is critical for the maintenance of cortical neural progenitor cells [15], and ephrin-B2/Fc promotes neuronal differentiation of adult NSCs in the hippocampus [16]. Considing the diversity of differenitaiton for different group, we quantified the fluorescence intensity of Tuj, a marker of immature neurons, to objectively assess whether ephrin-B2 has a similar function than ephrin-B1 in Fig. 2. Cells treated by siRNA-mediated knockdown of *efnb2* were compared to those treated with different doses of Ephrin-B2/Fc. The results showed that 2.5-ephrinB2$^+$ and ephrinB2$^-$ cells showed similar Tuj fluorescence intensity (576.3 ± 10.17 and 542.7 ± 6.06, respectively, compared to 474.3 ± 3.76 in the control group). Similar to a previous

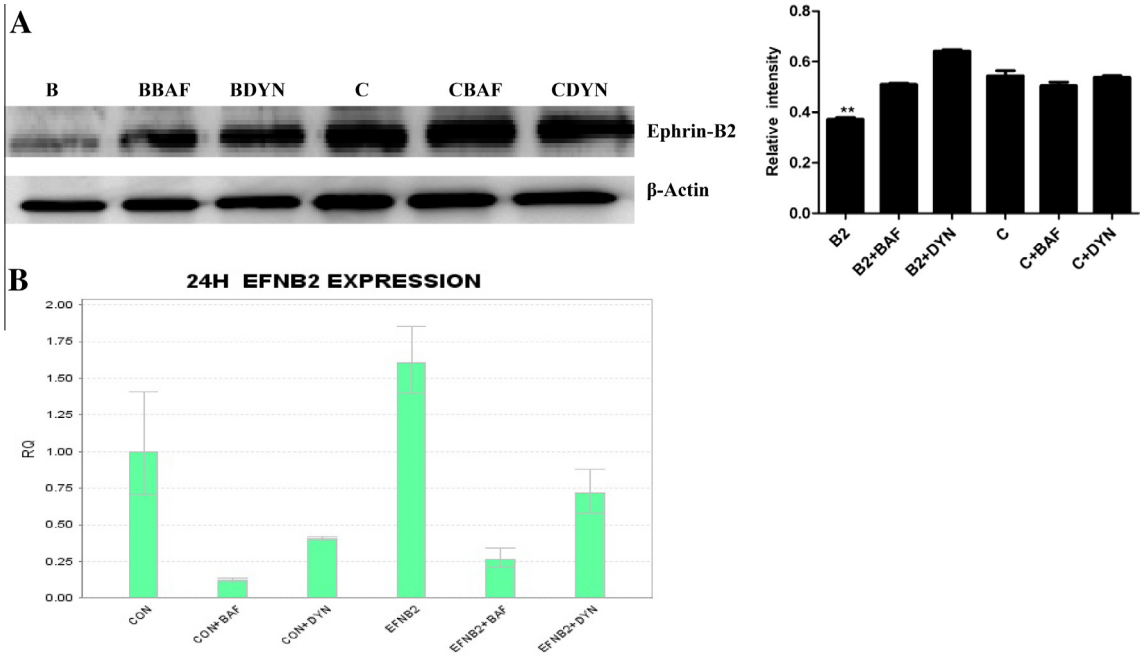

Fig. 3. Dynamic of ephrin-B2 during the ephrin-B2/FC treatment. (A) The expression of ephrin-B2 was decreased when treated with ephrin-B2/FC, but if coincubated with dynasore or bafilomycinA1, the expression of ephrin-B2 were no changing. (B) The mRNA expression of efnb2 were determined by real-time PCR, the expression of efnb2 was increased when treaed ephrin-B2/FC for 24 h. Dynasore or bafilomycinA1 could decreased the efnb2 expression. Data are the average ± S.E.M of triplicate by Applied Biosystems StepOne Software V2.1.

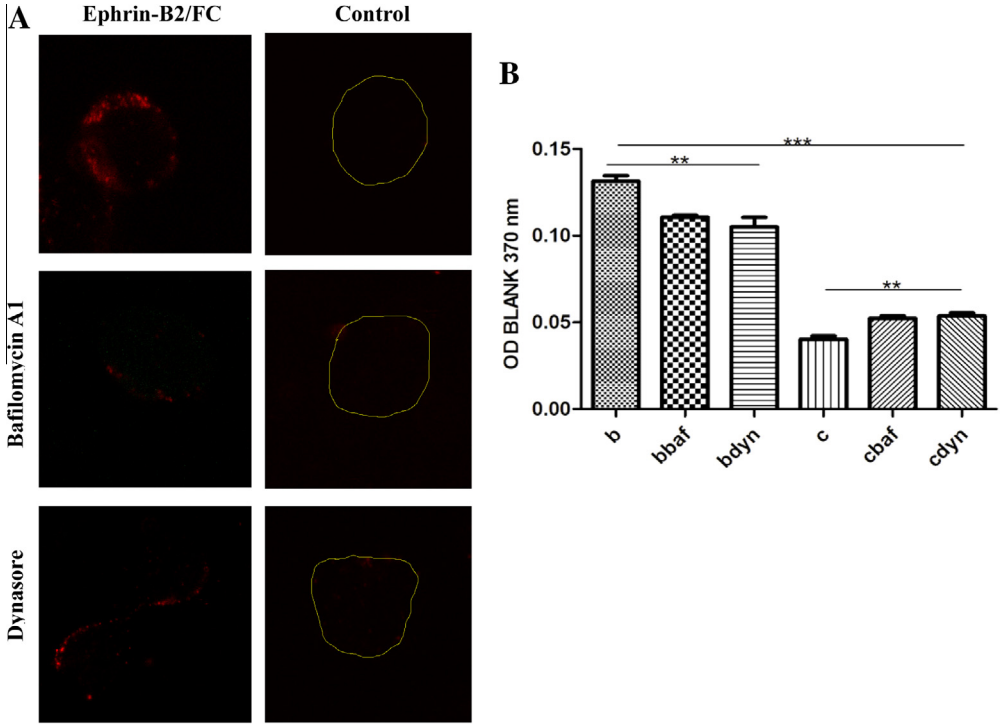

Fig. 4. Endocytosis and recycle of ephrin-B2/FC. (A) NSCs suface stably expressing exogenous ephrin-B2 when added ephrin-B2/FC after 1 h incubated, while blocked by dynasore or bafilomycinA1. (B) The level of ephrin-B2/FC recycle to the plasma membrane in NSCs was determined by ELISA. Data are the average ± S.E.M of independent experiments. $^{**}P < 0.01$; $^{***}p < 0.001$. (C) The EEA1 and human-IgG/FC were colocalization in NSCs when treated ephrin-B2/FC for 1 h. The ephrin-B2/FC was dropped or recycle decreased after using dynasore or bafilomycinA1. (D) Proposed model of dynamic of ephrin-B2/FC.

observation, the 0.5-ephrinB2$^+$ group (489.0 ± 10.54) did not show significant differences from the control group. These results indicate that ephrin-B2 inhibits neural progenitor cell differentiation.

A kinase-inactive mutant eph has been shown to cross-inhibit the activity of wild-type eph [17]. Therefore, to examine whether ephrin-B2/Fc fusion molecules could disturb endogenous ephrin-B2, we treated NSCs with recombinant ephrin-B2/Fc for 24 h and found that the ephrin-B2 level decreased. Interestingly, the expression of ephrin-B2 did not change when cells were co-incubated with dynasore or bafilomycin A1 (Fig. 3A). To further determine

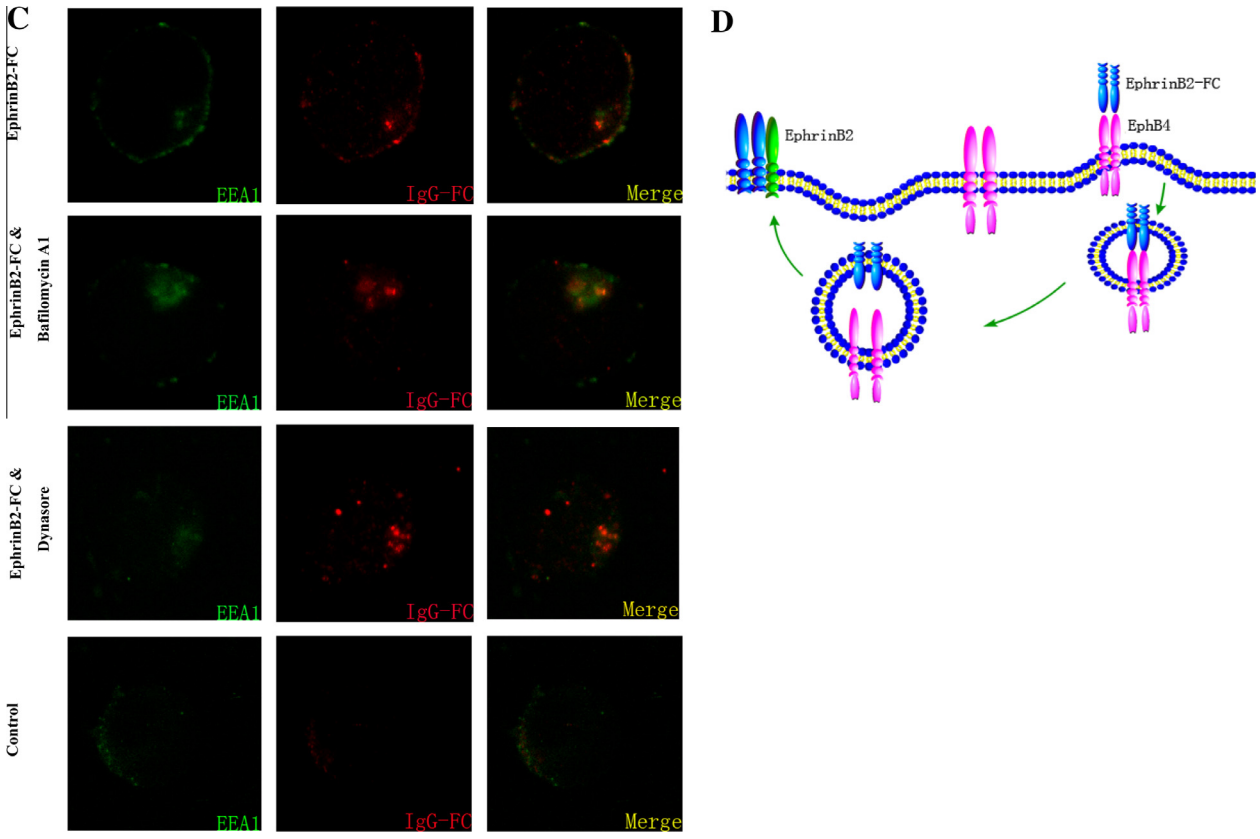

Fig. 4 (*continued*)

whether the down-regulation of *efnb2* mRNA expression caused the reduction of ephrin-B2 levels, NSCs were treated with recombinant ephrin-B2/Fc, which resulted in an increase in *efnb2* mRNA compared with the control group after 24 h. However, *efnb2* was decreased in the ephrin-B2/FC group and controls when cells were treated with dynasore or bafilomycin A1 (Fig. 3B). These results demonstrate that the exogenous ephrin-B2/FC would interferes with the endogenous ephrin-B2 and this effect occurs via endocytosis at the protein level. Furthermore, protein degradation can be rescued before the receptors recycle to the plasma membrane.

As the ephrin forward signaling cause the trans-endocytosis and eph would recycle to the plasma membrane after internalization [5]. we assumed that ephrin-B2/Fc could also recycle to the membrane and interfere with the endogenous ephrinB2 on the plasma membrane through cis inhibition. To gain insights into this hypothesis, we used surface labeling to analyze the movement of ephrin-B2/Fc [11,12]. We found that ephrin-B2/Fc was internalized and recycled to the plasma membrane, which was not observed in the control group. Furthermore, treatment of cells with dynasore or bafilomycin A1 reduced the surface fluorescence intensity in both groups. Therefore, the experimental process was corrected, and no false-positive results were obtained (Fig. 4A). To expand the number of samples, we used ELISA to assess the recycling of ephrin-B2/Fc [18]. We found a similar pattern of protein movement, confirming the data presented above (Fig. 4B). The EphrinB ligands reverse endocytosis to the membrane via a clathrin-mediated pathway [19]. To further address whether ephrin-B2/Fc was also in clathrin-coated pits, cells were stained with antibodies against the endosomal marker EEA1 and IgG/Fc secondary antibody. We found that EEA1 and IgG/Fc co-localized, and dynasore treatment inhibited this co-localization.(Fig. 4C). These results indicate that ephrin-B2/Fc is endocytosed via clathrin-coated pits and then recycled to the membrane.

The signal receptor gene transcription is fluctuateds dynamically and interfere by its intrinsic noise signal [20]. So a cell cannot generate a same output even input ligand is not differently [21]. Our findings demonstrate exogenous ephrin fusion protein have ability that enter intracellular and then recylce to plasma membrane to interfere the endogenous ephrin (Fig. 4D). This study provides insights that may help in the construction of the intracellular domain of the eph-ephrin ligand and shows its value as a molecular tool for the transfer of signals. This may facilitate strengthening of signals to overcome background noise in cells.

Acknowledgments

This work was supported by a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, and National Science Foundation of China Grant No. 81271367.

References

[1] D. Mukhopadhyay, H. Riezman, Proteasome-independent functions of ubiquitin in endocytosis and signaling, Science 315 (2007) 201–205.
[2] A. Sorkin, M. von Zastrow, Endocytosis and signalling: intertwining molecular networks, Nat. Rev. Mol. Cell Biol. 10 (2009) 609–622.
[3] E.B. Pasquale, Eph receptor signalling casts a wide net on cell behaviour, Nat. Rev. Mol. Cell Biol. 6 (2005) 462–475.
[4] A. Palmer, R. Klein, Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function, Genes Dev. 17 (2003) 1429–1450.
[5] M.E. Pitulescu, R.H. Adams, Eph/ephrin molecules – a hub for signaling and endocytosis, Genes Dev. 24 (2010) 2480–2492.
[6] J.C. Conover, F. Doetsch, J.M. Garcia-Verdugo, N.W. Gale, G.D. Yancopoulos, A. Alvarez-Buylla, Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone, Nat. Neurosci. 3 (2000) 1091–1097.
[7] R.S. Ashton, A. Conway, C. Pangarkar, J. Bergen, K.I. Lim, P. Shah, M. Bissell, D.V. Schaffer, Astrocytes regulate adult hippocampal neurogenesis through ephrin-B signaling, Nat. Neurosci. 15 (2012) 1399–1406.

[8] M. Aoki, T. Yamashita, M. Tohyama, EphA receptors direct the differentiation of mammalian neural precursor cells through a mitogen-activated protein kinase-dependent pathway, J. Biol. Chem. 279 (2004) 32643–32650.
[9] E.H. Allan, K.D. Hausler, T. Wei, J.H. Gooi, J.M.W. Quinn, B. Crimeen-Irwin, S. Pompolo, N.A. Sims, M.T. Gillespie, J.E. Onyia, T.J. Martin, EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts, J. Bone Miner. Res. 23 (2008) 1170–1181.
[10] H. Li, G. Jin, J. Qin, W. Yang, M. Tian, X. Tan, X. Zhang, J. Shi, L. Zou, Identification of neonatal rat hippocampal radial glia cells *in vitro*, Neurosci. Lett. 490 (2011) 209–214.
[11] S. Yoo, Y. Kim, H. Noh, H. Lee, E. Park, S. Park, Endocytosis of EphA receptors is essential for the proper development of the retinocollicular topographic map, EMBO J. 30 (2011) 1593–1607.
[12] M. Zimmer, A. Palmer, J. Kohler, R. Klein, EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion, Nat. Cell Biol. 5 (2003) 869–878.
[13] T. Kirchhausen, E. Macia, H.E. Pelish, Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis, Small Gtpases Dis. 438 (2008) 77–93.
[14] S.J. Kotowski, F.W. Hopf, T. Seif, A. Bonci, M. von Zastrow, Endocytosis promotes rapid dopaminergic signaling, Neuron 71 (2011) 278–290.
[15] R. Qiu, X. Wang, A. Davy, C. Wu, K. Murai, H. Zhang, J.G. Flanagan, P. Soriano, Q. Lu, Regulation of neural progenitor cell state by ephrin-B, J. Cell Biol. 181 (2008) 973–983.
[16] R.S. Ashton, A. Conway, C. Pangarkar, J. Bergen, K.I. Lim, P. Shah, M. Bissell, D.V. Schaffer, Astrocytes regulate adult hippocampal neurogenesis through ephrin-B signaling, Nat. Neurosci. 15 (2012) 1399–1406.
[17] P.W. Janes, B. Griesshaber, L. Atapattu, E. Nievergall, L.L. Hii, A. Mensinga, C. Chheang, B.W. Day, A.W. Boyd, P.I. Bastiaens, C. Jorgensen, T. Pawson, M. Lackmann, Eph receptor function is modulated by heterooligomerization of A and B type Eph receptors, J. Cell Biol. 195 (2011) 1033–1045.
[18] A. Morcavallo, M. Genua, A. Palummo, E. Kletvikova, J. Jiracek, A.M. Brzozowski, R.V. Iozzo, A. Belfiore, A. Morrione, Insulin and insulin-like growth factor II differentially regulate endocytic sorting and stability of insulin receptor isoform A, J. Biol. Chem. 287 (2012) 11422–11436.
[19] M. Parker, R. Roberts, M. Enriquez, X. Zhao, T. Takahashi, D.P. Cerretti, T. Daniel, J. Chen, Reverse endocytosis of transmembrane ephrin-B ligands via a clathrin-mediated pathway, Biochem. Biophys. Res. Commun. 323 (2004) 17–23.
[20] N. Rosenfeld, J.W. Young, U. Alon, P.S. Swain, M.B. Elowitz, Gene regulation at the single-cell level, Science 307 (2005) 1962–1965.
[21] R. Cheong, A. Rhee, C.J. Wang, I. Nemenman, A. Levchenko, Information transduction capacity of noisy biochemical signaling networks, Science 334 (2011) 354–358.